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13. ABSTRACT (Maximum 200 Words)

In order to kill prostate tumor cells without affecting non-tumor tissues using gene therapy, it is essential to deliver therapeutic genes specifically to the tumor cells. Gene delivery vehicles can be targeted to specific cell types by screening libraries of retroviruses displaying random peptides in the cell-targeting region of the envelope protein (Env). The retroviruses are screened for their ability to deliver a selectable marker gene to the target cell. A library displaying 2 x 10^7 different peptides within the envelope protein has been screened on the human PC-3 and DU145 prostate cell lines. Several Env proteins have been obtained that can deliver a gene to the PC-3 cell line. To date, no Env isolate has been successfully identified after screening on the DU145 cell line. Further characterization of the PC-3 targeted Env proteins continues. These proteins promise to be useful reagents in the treatment of prostate cancer.

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INTRODUCTION

Gene therapy is a newly developing approach for the treatment of prostate cancer. Since the genes to be delivered to tumor cells eventually lead to cell death, it is critical that the genes not be delivered to non-prostate cells. Retroviral entry into cells is controlled by the surface envelope (Env) protein and can be altered by replacement of a 10 amino acid peptide sequence in the cell-targeting region. By screening libraries of retroviral Env proteins with random peptides substituted into this region, novel Env isolates that productively deliver a gene to PC-3 human prostate tumor cells have been identified. These targeted Env proteins will be important reagents in the development of gene therapy treatments for prostate cancer.

BODY

Summary of Research Findings

Task 1

The basic method used in these studies is to generate a large constitutive library of Env proteins with random substitutions within the receptor-binding domain and score for productive infection on specific prostate cell lines. To this end, a stable constitutive library of 2×10^7 different Env variants was created as proposed. In our initial screening on 143B osteosarcoma cells, the number of positive isolates was extremely limited (one isolate). A modification of the screening process was therefore tested on the prostate cells. This modification introduced a replication competent amphotropic 4070A retrovirus into the library, with the idea that amphotropic Env could assist in the initial passage of a suboptimal Env isolate. This amphotropic Env would assist in the enrichment and amplification of the functional Env proteins within the library by allowing these variants to spread throughout a population of infected cells. Entry using the amphotropic receptor, though, is not the aim of the study. Therefore, entry through the amphotropic receptor was limited through the use of receptor interference. Pre-infection of the target prostate cells with amphotropic virus downregulates the amphotropic receptor. Using this process, the background of infection via the amphotropic receptor is limited.

One prediction of using the mixed infection of the Env library with replication competent virus is that additional rounds of selection would be required to identify a unique novel prostate-cell targeting isolate. Seven separate 150mm diameter plates of 4070A-infected PC-3 cells were inoculated with 1 liter of the library supernatant and then selected for G418 resistance. As expected, thousands of G418 resistant colonies were obtained. Filtered supernatant from these G418 resistant cells was then used to inoculate a fresh population of 4070A-infected PC-3 cells in seven separate plates for a secondary round of screening. After 2 days, the cells from each plate were divided into two groups. One group was placed immediately under selection for G418 resistance. The other group was

passaged in the absence of the drug for one month. This allowed the most efficient PC-3 targeting Env proteins to spread throughout the culture.

From the population of cells that was placed under direct selection for drug resistance, at least 3 separate plates contained Env proteins exhibiting properties of PC-3-targeted Env proteins. These plates consist of small pools of random variants. Viral titers are more rapidly determined using lacZ as the transfer gene. Therefore, these pools of Env variants were transferred into the TELCeB6 cell line that expresses the lacZ marker within a retroviral vector. Virus released from these cells would express the novel Env on the surface of retroviral particles and package the gene for the *lacZ* marker. When the pool of retroviral particles from plate PA2 was applied to 4070A-infected PC-3 cells and *lacZ* transfer measured two days later, the titer was $2.1 \pm 0.6 \times 10^2$ ml^{-1}. The titer mediated by control 4070A-infected TELCeB6 cells was 5 ± 4 ml^{-1} (n=3). Two additional plates also yielded titers above background. The tropism of these Env proteins is therefore distinct from the parental FeLV-A Env protein, which is unable to infect PC-3 cells (Bupp and Roth, manuscript submitted).

Even more promising results were obtained using virus harvested from the cells that had been passaged for one month in the absence of drug and then selected for G418 resistance. Viruses from six plates were separately transferred to the TELCeB6 *lacZ* marker cell line and tested on 4070A-infected PC-3 cells. The titers obtained in this case ranged from 2.8×10^2 to 8.95×10^2 per ml depending on the plate. Infection of the target cells by control 4070A-infected TELCeB6 cells was undetectable (<4 per ml). Thus, PC-3 targeting Env proteins were enriched in these populations compared with the population that underwent direct selection for G418 resistance.

Env sequences from these pools are presently being PCR amplified and cloned to further characterize their cell-targeting specificity as well as titers in the absence of 4070A virus and other non-functional library Env variants. No DU145-targeted Env proteins were obtained by screening the library on DU145 cells.

Task 2

We have not vigorously pursued the inverse targeting approach. This approach was initially, not enthusiastically supported by the reviewers and the approach is risky. The STEAP protein and the prostate specific membrane antigen (PSMA) were both proposed as possible targets using this approach. So far, all mammalian retroviruses require multipass membrane proteins as receptors. The STEAP protein therefore appears to have a structure appropriate for mediating retroviral entry. However, we have been unable to obtain a clone encoding this protein from UroGenesys, Inc. who appear to be the sole source of this reagent, despite two requests for the reagent. Alternative sources for the gene are now being considered.

KEY RESEARCH ACCOMPLISHMENTS

1. Libraries of retroviruses expressing 2 x 10^7 different peptides within the cell targeting region of the Env protein have been screened for the ability to deliver a selectable marker to human PC-3 and DU145 prostate tumor cell lines.

2. Several Env variants targeting PC-3 cells have been enriched from the library by screening on the PC-3 cell line.

REPORTABLE OUTCOMES

Oral presentation at Cold Spring Harbor Laboratory "Vector Targeting Therapeutic Strategies for Gene Therapy" meeting (2003): K. Bupp and M. Roth, "Targeting retroviruses using random peptide display libraries."

Poster presentation at Cold Spring Harbor Laboratory "Retroviruses" meeting (2003): Keith Bupp, Xuejun Ma and Monica J. Roth, "Random Mutational Analyses of the receptor-binding domain of feline leukemia virus."

A manuscript entitled "Targeting a retroviral vector in the absence of a known cell targeting ligand" by Keith Bupp and Monica J. Roth has been submitted.

The results of this work form the basis of a New Investigator Award application (PC030516) to the USAMRMC-CDMRP by Keith Bupp entitled "Development of a mouse model for prostate cancer gene therapy using retroviral vectors targeting prostate cells."

CONCLUSIONS

Gene therapy treatment of both localized and malignant prostate cancer will require the use of prostate-targeted gene therapy vectors. The retroviral Env proteins identified during the course of this work are promising reagents to be used to target gene delivery to prostate cells.

REFERENCES: None

APPENDICES:

1) Copy of manuscript, Keith Bupp and Monica Roth
2) Abstract of presentation at Retrovirus meeting at Cold Spring Harbor, NY May 2003.
3) Abstract of presentation at "Vector Targeting Therapeutic Strategies for Gene Therapy" meeting at Cold Spring Harbor, March, 2003.

TARGETING RETROVIRUSES USING RANDOM PEPTIDE DISPLAY LIBRARIES

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We have developed a new technology that can be used to retarget retroviruses for gene therapy. It involves screening libraries of subgroup A feline leukemia virus (FeLV-A) Env proteins with random amino acids substituted into the cell-targeting region. The randomly substituted Env proteins constituting the library are expressed on the surface of retroviral particles from bicistronic, packageable retroviral cassettes carrying both the *env* gene and the *neo* gene. The libraries are constitutively expressed from stable producer cells. Screening libraries on canine D17 cells and human 143B cells yielded Env proteins specific for canine (D17) and human (143B, 293T) cells, respectively. In contrast, screening libraries on feline AH927 cells yielded Env proteins that infected AH927, 143B, 293T and, to a much lower extent, D17 cells. Sequence analyses of the receptor-determining regions indicated very little conservation among the random peptides conferring similar tropic properties. The feline leukemia viruses are divided into the A, B and C subgroups based on interference assays. FeLV-A preferentially infects feline cells while FeLV-B and FeLV-C have broader host ranges. Superinfection interference assays indicated that canine-specific Env proteins were using the FeLV-C receptor. However, neither the human-selected nor the feline-selected clones used the FeLV-A, -B or -C receptor. They also did not use the receptor for the amphotropic 4070A virus. Thus, random-display Env libraries can be used to target retroviruses using novel receptors. The technique promises to be very useful for targeting specific cells using gene therapy vectors.

RANDOM MUTATIONAL ANALYSES OF THE RECEPTOR-BINDING DOMAIN OF FELINE LEUKEMIA VIRUS

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We have investigated the importance of two conserved residues within the receptor-binding domain of feline leukemia virus (FeLV) Env proteins by generating libraries of random mutants at these residues. We had previously isolated a derivative of FeLV-A that infects D17 canine cells via an FeLV-C receptor but did not infect feline cells in contrast to the parental virus. The derivative (B82) was obtained by screening a library of FeLV-A Env derivatives with random amino acids substituted at ten positions in the receptor-determining region. The random amino acid substitutions surrounded a highly conserved positively charged residue that was held constant in the screen. In order to test the importance of this residue, 13 different amino acids were substituted into this position. In infectivity assays, charged residues (R,H,D,K) were preferred at this position, followed by residues with a hydrophilic component (N,Y,S,T). Hydrophobic substitutions (W,A,P,V,L) and cysteine were not well tolerated at this position. Thus, maintaining a positively charged residue at this position may have biased the screen to target a natural FeLV receptor.
A second conserved residue was similarly examined. Located 17 residues upstream of the proline-rich region in the primary sequence, Y183 is absolutely conserved among FeLV-A, -B and -C Env but not with MuLV Env proteins. A computer-generated model of FeLV-A Env indicates that Y183 is protruding from the surface. These observations suggest its possible role in receptor binding. A random library was generated at this position within the FeLV-A 61E isolate. In order to do this oligonucleotides containing degenerate sequences at position 183 were ligated into a newly created restriction enzyme site. 12 different amino acids were substituted into this position. Each of these clones was individually transfected into TELCeB6 cells and *lacZ* gene transfer to feline AH927 cells was determined. Titering results demonstrated that FeLV-A maintained full infectivity to AH927 cells when Y was changed to either E or W while losing its infectivity to undetectable levels when Y was changed to either G, C, T or N. Substitution by R, F, S, V, L, D yielded intermediate levels of infectivity. This suggests that the Y183 might have functional significance for virus binding and entry.

Targeting a Retroviral Vector in the Absence of a Known Cell-Targeting Ligand

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ABSTRACT

An important requirement for gene transfer vectors is to direct therapeutic genes specifically to target cells. Here we describe an improved targeting method that does not depend upon the use of a known cell-targeting ligand. It entails screening a library of constitutively produced retroviruses with random amino acid substitutions in the cell-targeting region of the envelope proteins for their ability to mediate gene delivery to a target cell. By screening such a library on the *ras*-transformed 143B human cell line, we have isolated an envelope protein that preferentially targets 143B cells and 293T cells expressing the SV40 T antigen via a novel, unidentified receptor. Furthermore, retroviruses expressing the library-derived envelope protein can be concentrated by centrifugation. This represents a major advance in vector targeting: the selection of productive retroviral entry via an alternate receptor with a novel cellular tropism in the absence of a known cell-targeting moiety. The method is applicable even to cells that have not been well characterized, and therefore is suitable for targeting many diverse cell types.

OVERVIEW SUMMARY

This study develops a library screening strategy for targeted delivery of retroviral vectors to specific cell types, circumventing the requirement for known cell-targeting ligands. A library of retroviruses expressing random peptides in the cell-targeting region of the feline leukemia virus envelope (Env) protein was screened for Env proteins that could mediate gene delivery to the *ras*-transformed 143B human cell line. One Env protein variant was able to infect these cells and 293T cells expressing the SV40 T antigen. Interestingly, the parental cell lines for 143B and 293T, not expressing the viral oncogenes, were less sensitive to transduction by the same Env protein. Gene delivery to several other cell lines was either low or undetectable. Receptor interference assays revealed that the 143B-targeted Env protein did not use the FeLV receptors or the amphotropic 4070A receptor. This approach is highly versatile, yielding functional viral entry through a novel host-cell receptor.

INTRODUCTION

Gene therapy is a promising approach for treating cancer. A number of different strategies involving diverse genes are currently being investigated (Dachs *et al.*, 1997). These include the delivery of toxic genes (Martin *et al.*, 2000), immuno-modulatory genes (Hull *et al.*, 2000), genes for pro-drug converting enzymes (Hughes *et al.*, 1995; Koeneman *et al.*, 2000) as well as others (Galanis *et al.*, 2001). The ability to deliver these genes specifically to tumor cells would be an important advancement. One of the challenges of targeting retroviral vectors has been to translate the binding properties of a previously characterized cell-targeting molecule into a successful virus-cell fusion event, followed by stable gene delivery. Several such targeting strategies have been tested (Bupp and Roth, 2000; Lavillette *et al.*, 2001; Liu *et al.*, 2000; Martin *et al.*, 2002; Morizono *et al.*, 2001; Peng *et al.*, 1997; Snitkovsky *et al.*, 2000). In the most common approach, a chimeric protein is constructed between a targeting ligand or antibody and the retroviral surface envelope (Env) protein (Bupp and Roth, 2000; Lavillette *et al.*, 2001). However, such covalent Env-ligand hybrids frequently fail to be incorporated into viral particles or to mediate fusion (Benedict *et al.*, 1999; Cosset *et al.*, 1995a; Schnierle *et al.*, 1996; Zhao *et al.*, 1999).

In order to avoid the gross structural changes in Env resulting from hybrid Env-ligand constructs, we have developed a strategy involving the simple substitution of random peptides into the cell-targeting region. Libraries of these substituted Env proteins expressed on the surface of retroviral particles are then screened for their ability to mediate stable delivery of a drug resistance marker to target cells. Only the derivatives that can fulfill all the essential requirements for virion incorporation, cell-binding and fusion are thereby recovered. The method enables a vector to be targeted to a cell without prior knowledge of its complement of cell-surface proteins. In addition, well-characterized cells could also be targeted when known cell surface proteins fail to serve as efficient retroviral receptors.

The library is based on the feline leukemia virus subgroup A (FeLV-A) Env (Donohue *et al.*, 1988; Overbaugh *et al.*, 1988) which preferentially mediates infection of feline cells. We previously used a transiently produced retroviral library to isolate a FeLV-A variant that specifically infected a canine osteosarcoma cell line, utilizing a receptor that is naturally bound by the FeLV-C Env (Bupp and Roth, 2002). The results presented herein describe the successful targeting of the Ki-*ras* transformed 143B human osteosarcoma cell line using a non-FeLV receptor. The ability to target a retrovirus to a previously unidentified

alternate receptor demonstrates the versatility of the library-screening approach for cell-specific targeting.

MATERIALS AND METHODS

Cell lines

143B (provided by F. González-Scarano) and AH927 (provided by J. Overbaugh) were grown in MEM + 10% fetal calf serum (FCS). HOS, A-498 (both from ATCC, Manassas, VA), Huh-7, and HeLa (both provided by J. Dougherty) were grown in MEM + 10% FCS with 1 mM sodium pyruvate and 0.1 mM non-essential amino acids (GibcoBRL/Invitrogen Carlsbad, CA). 293, TE671, RD (all 3 from ATCC), WI38 (provided by K.-Y. Chen), and DLD-1 (provided by K. Chada) were grown in DMEM + 10%FCS. 293T were grown in DMEM + 10% FCS with 400 μg per ml G418. The presence of T antigen in the cell line was confirmed by PCR analysis (data not shown). PC-3 (provided by C. Abate-Shen) were grown in RPMI medium 1640 containing 25 mM Hepes + 10% FCS and non-essential amino acids. TELCeB6 cells (Cosset *et al.*, 1995b) (provided by Y. Takeuchi) were grown in DMEM + 10% FCS containing 6 μg/ml blasticidin S (ICN, Costa Mesa, CA). All media contained an antibiotic/antimycotic mixture (GibcoBRL/Invitrogen).

Plasmid library construction

The library of plasmids containing *env** genes with degenerate oligonucleotides was constructed by scaling up the previously described

method (Bupp and Roth, 2002) using 6.9 μg instead of 700 ng of the BbsI-cut pRVL vector. Greater than 95% of the 3 x 10^7 transformants resulting from the ligation of pRVL with degenerate oligonucleotides were shown by PCR and sequence analysis to contain random nucleotide sequences in the cell-targeting region. The complexity of the plasmid library was therefore 3 x 10^7.

Generation and screening of a retroviral library of Env proteins*

To generate a cell line constitutively producing a library of Env* proteins expressed on retroviral particles, 100 μg of library DNA was transfected by calcium phosphate precipitation (Stratagene, La Jolla, CA) into ten 10 cm dishes of 30% confluent 293T*gagpol* cells (Bupp and Roth, 2002) along with 100 μg pHIT-G (Fouchier *et al.*, 1997) (provided by M. Malim) in the presence of 12.5 μg per ml chloroquine. The gene encoding VSV-G cannot be incorporated into retroviral particles from this construct. After 16 hours the medium was changed and sodium butyrate was added to 10 mM and the cells were incubated 8 hours at 37°. The cells were then rinsed with phosphate buffered saline and DMEM/10% FCS was added. 24 hours later the supernatant was harvested, filtered and added to 4 x 10^7 143B*gagpol* cells in the presence of 8 μg/ml polybrene. 143B*gagpol* cells are a clone of 143B cells stably transfected with the CeB plasmid (Cosset *et*

al., 1995b) (provided by Y. Takeuchi) and selected with 6 μg/ml blasticidin S. After an overnight incubation, the medium was changed to remove polybrene. 36 hours later the cells were split into medium containing 400 μg/ml G418 to eliminate non-transduced cells. No cells were discarded. A parallel control titration of the supernatant from the transfected 293T*gagpol* cells indicated that it contained 3 x 10^5 total *neo*-transducing particles on 143B cells.

Confluent producer cells were split 1:8 into twenty 10 cm dishes in the absence of G418. 3 days later, half of the 180 ml supernatant was harvested, filtered through a 0.45 μm filter and used to inoculate 30% confluent 143B*gagpol* cells in a 150 mm plate in the presence of 8 μg per ml polybrene. After overnight incubation, medium was changed to remove polybrene. 36 hours later, cells were split into medium containing 400 μg per ml G418. One clone was obtained. In an independent control experiment, the number of retroviral particles in the 143B*gagpol* producer cell supernatant when the parental FeLV-A Env (R5FeLV-A (Bupp and Roth, 2002)) was used in place of randomly substituted *env** genes was found to be 3 x 10^4 per ml on AH927 cells. The 90 ml of supernatant used for screening on target cells therefore corresponded to approximately 3 x 10^6 wild-type infectious particle equivalents or 10 particles per random variant.

To transfer the cassette encoding the L1 *env* gene from the G418^R 143B*gagpol* clone, the cells were transfected with pHIT-G (Fouchier *et al.*, 1997) and induced with sodium butyrate as described above for generation of the library. The supernatant was then harvested, filtered and used to inoculate TELCeB6 cells (Cosset *et al.*, 1995b) in the presence of 8 μg/ml polybrene. Medium was changed to remove polybrene after 16 hours and 32 hours later the cells were split into medium containing 400 μg/ml G418.

Titer determination and interference assays

Titration analyses and interference assays were performed as previously described (Bupp and Roth, 2002). L1 pseudotyped virus was concentrated by overnight centrifugation in an SW27 rotor at 9,500 rpm. For interference assays and results in Table 2, results presented are representative of at least 2 separate experiments, each in duplicate. For other titrations, results are averages ± standard deviations from at least two separate experiments, each in duplicate. Derivatives used for interference were made by transfection of the following proviral DNAs: p61E (FeLV-A) (Donohue *et al.*, 1988; Overbaugh *et al.*, 1988) provided by J. Mullins through the NIH AIDS Research & Reference Reagent Program (Rockville, MD), EESS (FeLV-C (Riedel *et al.*, 1988)) also provided by J.

Mullins, EEZZ (FeLV-B (Boomer *et al.*, 1997)) provided by J. Overbaugh, and pNCAC-Am (amphotropic 4070A MuLV) (Peredo *et al.*, 1996).

The 143B cells expressing L1 Env were generated essentially as previously described for the generation of D17 cells expressing EF Env (Bupp and Roth, 2002). The oligonucleotide encoding the random peptide sequence of L1 (5'-GTG GGA GAC ACC TGG GAA CCT TAC TGG CTT CCA GCC AGA AGT CAC AAT ATC GCG TCC TCC TCA AAA TAT GGA-3') was ligated back into the pRVL library vector to express L1 Env as described for pRVEF1 (Bupp and Roth, 2002). The ten residues that were randomized for the library are underlined. Transfer of the resulting retroviral vector into 143B cells following transient transfection into 293TCeB cells was as described (Bupp and Roth, 2002). 8 143B/L1 clones were screened for their ability to interfere with subsequent infection by L1. All 8 clones showed significant interference. Clone #1 was used for the analyses reported in Table 6.

RESULTS

Overview of the random library screening approach

The basic strategy for obtaining Env proteins with novel targeting properties is to screen a population of retroviruses with random amino acid substitutions in the cell-targeting region of the Env protein. These Env variants are expressed on the surface of retroviruses and are tested for their ability to mediate stable delivery of a drug resistance marker to target cells. If the Env protein is functional, the gene encoding it can be characterized from the drug-resistant colony, since it is linked to the drug-resistance marker on the retroviral gene delivery cassette (Fig. 1A).

Generation and screening of a constitutively expressed random Env library

In order to facilitate Env library screening, a constitutive library producer cell line was generated to provide a long-term stable source of the library and eliminate variability between independent transient transfection procedures (Bupp and Roth, 2002). Three steps are required to generate and screen a constitutive Env library (Fig. 1). The first is to construct a plasmid library of mutated *env* genes (*env**) coding for Env* proteins with random amino acids in the cell-targeting region (Fig. 1A). The modified FeLV-A 61E (Donohue *et al.*, 1988; Overbaugh *et al.*, 1988) *env** genes are expressed upstream of a G418 selection marker on the bicistronic

retroviral cassette. As a result of incorporating equal mixtures of all four nucleotides at each position of ten codons, the Env* proteins contain random amino acid substitutions at those ten positions within the cell-targeting region.

Next, a constitutive retroviral producer cell line is generated from the plasmid library (Fig. 1B). The plasmid library consisting of 3 x 10^7 different *env** derivatives was transiently transfected together with the plasmid pHIT-G (Fouchier *et al.*, 1997) encoding the vesicular stomatitis virus (VSV) G protein into 293T*gagpol* cells (Bupp and Roth, 2002) (Fig. 1B). These cells express the murine leukemia virus (MuLV) Gag and Pol retroviral core proteins. The resulting supernatant contained retroviral particles expressing the VSV-G glycoprotein on their surfaces and packaging the library cassettes. The use of the VSV-G protein allows for the efficient, stable introduction of the library into the acceptor cells independent of the randomized Env* function. Supernatant containing the VSV-G coated particles was used to inoculate 143B*gagpol* cells constitutively expressing Gag and Pol proteins. Infection was performed at an MOI of 0.01 to ensure that only a single retrovirus, packaging one dimer of viral vector, would infect a given cell.

The resulting $G418^R$ cells constitutively produced retroviral particles expressing the randomly substituted Env* proteins on their surfaces (Fig. 1C). These particles also packaged the cassettes encoding the genes for the

corresponding surface-expressed Env* variants. A control titration of G418^R transfer from the transiently transfected 293T*gagpol* cells indicated that the complexity of the library of retroviral cassettes was approximately 3 x 10^5 different variants. Thus, the retroviral library expressed from the producer cells represented 1% of the plasmid library.

Finally, viral supernatant from the 143B*gagpol* cells constitutively producing the Env* library was tested for targeting. Here the supernatant was tested on 143B*gagpol* cells (Fig. 1C). The use of 143B cells as both producers and targets does not bias the outcome. Successful screening of alternative target cell lines has also been performed using virus from the 143B-based producer cells (K. Bupp and M. Roth, manuscript in preparation). A single colony was obtained after G418 selection of library-screened 143B cells. The *env**-encoding cassette from this clone (L1) was then transferred to TELCeB6 cells (Cosset *et al.*, 1995b). The TELCeB6 cell line expresses Gag and Pol proteins as well as a packageable *lacZ* marker cassette, allowing rapid quantitation of L1 Env-mediated gene transfer.

L1 infects human 143B and 293T cells expressing viral oncogenes

The titer of L1-pseudotyped particles from TELCeB6 cells was examined on a battery of human cell lines derived from various tissue origins and also cells from other species (Table 1). High titers were observed only on human 143B cells, the target cell of the initial screen (8.4 x 10^3 per ml),

and 293T cells (1.9 x 10^5 per ml). Infection of other cell lines by L1, including feline, canine and murine cells, was either minimal or undetectable. L1 was therefore restricted both in its species and cellular tropisms. As expected, the parental FeLV-A Env gave a very high titer (5.0 x 10^6 per ml) on AH927 feline cells and FeLV-C was able to mediate transduction on almost all cell types tested. In contrast to L1, the titer of the parental FeLV-A Env was extremely low on the 143B cells (2 x 10^1). Thus, the tropism of L1 was quite distinct from the parental FeLV-A and the FeLV-C Env proteins.

Interestingly, a dramatic difference of nearly four orders of magnitude was observed when the titer of L1 on 293T cells (DuBridge *et al.*, 1987) was compared with the titer on 293 cells (Graham *et al.*, 1977) (Table 1). By comparison, the titers of FeLV-A and -C were only slightly reduced. In order to obtain higher titers of L1, supernatant from TELCeB6/L1 clone 29 was subjected to ultracentrifugation. This resulted in a 31-fold concentration with 81% yield to 2.1 x 10^5 per ml on 143B cells (Table 2). Remarkably, testing the same supernatant on the parental HOS cell line showed an approximately 50-fold decrease in titer. In contrast, titers of FeLV-B and FeLV-C remained similar on both cell lines. 143B cells were derived from HOS cells by infection with Kirsten mouse sarcoma virus (Campione-Piccardo *et al.*, 1979; Rhim, 1981; Rhim *et al.*, 1975). Thus,

oncogene expression in 293T and 143B cells correlated with increased L1 infection.

L1 does not use a FeLV receptor

The receptor usage of the L1 variant was examined by performing interference assays, in which expression of an Env protein within a cell can block the subsequent infection of a virus utilizing the same viral receptor. Greater than 99% inhibition of infection was considered to be significant interference (Chesebro and Wehrly, 1985; Rein, 1982). As shown in Table 3, preinfection of 143B cells with FeLV-C did not prevent subsequent infection of those cells by L1. The titer of FeLV-A on 143B cells was too low to test for interference. However, preinfection of 293T cells with either FeLV-A or FeLV-C did not prevent subsequent infection by L1-pseudotyped particles (Table 4). The diminution in titer by L1 on the FeLV-C infected cells was likely due to the adverse effect of the FeLV-C infection on this cell line. Preinfection of 293T cells by FeLV-B or the amphotropic murine leukemia virus (MuLV) 4070A also did not prevent subsequent infection by L1 (Table 5). In these experiments, FeLV A, B, C and MuLV 4070A each individually blocked challenge by the cognate virus.

To further confirm the usage of a novel receptor by L1, the L1 Env protein was expressed in 143B cells. Subsequent challenge by L1, FeLV-B, FeLV-C or 4070A confirmed results of the previous experiments (Table 6).

L1 inhibited subsequent gene transfer only by L1-expressing retroviral particles but not particles expressing the other three Env proteins on their surfaces. Altogether these results indicate that the receptor used by L1 is neither a FeLV receptor nor the 4070A amphotropic MuLV receptor.

Sequence of L1 Env

The sequence of the surface (SU) portion of L1 Env was obtained from PCR amplification products of genomic DNA (Bupp and Roth, 2002) from the TELCeB6 L1 derivative. SU contains the receptor-binding domain at its N-terminus. The only changes in L1 Env compared with the parental FeLV-A were the ones introduced into the randomized region by oligonucleotide mutagenesis. The sequence of this region in L1, WEPYWLPARSHNIASSS (see Fig. 1A), exhibits very little similarity to wild-type FeLV-A or FeLV-C isolates (not shown). A BLAST search (Altschul *et al.*, 1990) of the protein database failed to identify any relevant sequences homologous to the peptide from the randomized region of L1.

DISCUSSION

By screening a random peptide-display library of FeLV Env proteins we have targeted a retroviral vector to two cell lines expressing viral oncogenes. Of the fifteen cell lines tested, efficient gene transfer mediated by the L1 Env protein was only observed on 143B and 293T cells. In contrast, the parental FeLV-A Env protein had a low titer on 143B cells. Most strikingly, interference assays indicated that L1 no longer used any FeLV receptor. The ability to target retroviral entry in the absence of a known cell-targeting moiety is in contrast to previous strategies that have used characterized ligands or antibodies to target specific cell types (Bupp and Roth, 2000; Lavillette *et al.*, 2001). Screening a library of Env proteins for stable gene transfer circumvents the obstacles faced when attaching a known targeting ligand to an Env protein for retargeting. Library-selected Env proteins must necessarily be able to carry out all of the important functions of Env critical for cell binding and fusion.

The nature of the receptor utilized by L1 is unknown. Interference assays indicate that the receptors used by FeLV-A, -B, and -C and amphotropic MuLV 4070A are distinct from that bound by L1. Three out of four of these receptors have been cloned. The receptors used by FeLV-B and MuLV 4070A are the closely related phosphate transporters Pit1 and Pit2, respectively (Kavanaugh *et al.*, 1994; Takeuchi *et al.*, 1992; van Zeijl

et al., 1994). The receptor for FeLV-C is homologous to organic anion transporters (Quigley *et al.*, 2000; Tailor *et al.*, 1999). The fact that L1 does not infect human TE671 cells also suggests that it is not using either the xenotropic MuLV receptor or the RD114 feline endogenous retrovirus receptor. TE671 cells are readily infected by retroviruses using either of these receptors (Tailor *et al.*, 1999a; Tailor *et al.*, 1999b).

The mechanism by which the SV40 T antigen and Ki-*ras* protein affect the entry properties of L1 remains to be determined. It is possible that the L1 receptor is itself regulated by the Ki-*ras* gene product and/or the SV40 T antigen. Identification of the L1 receptor will help to clarify the relationship. However, it should be noted that determinants in addition to the T antigen and Ki-*ras* protein are required for the induction of the L1 receptor. The DLD-1 colon carcinoma and PC-3 prostate tumor cell lines both express activated Ki-*ras* oncogenes (Pergolizzi *et al.*, 1993; Shirasawa *et al.*, 1993) and yet are not readily susceptible to L1-mediated gene transfer. Since cellular transformation is complex and requires activation and inhibition of multiple pathways (Lynch and de la Chapelle, 1999; Trapman, 2001), it is not surprising that other factors are also involved in susceptibility to L1.

The results reported here establish that the library screening approach for retroviral retargeting to novel receptors can potentially be developed for gene therapies directed to any given cell type. The ability to

identify cell-surface markers unique to oncogene-expressing cells is also a major advance in defining cellular transformation. Once identified, the receptor used by L1 may be useful in immuno-therapeutic approaches to cancer treatment. The ability to isolate such an Env protein from a random peptide-display library demonstrates the significant versatility and potential for this retargeting method.

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TABLE 1. COMPARISON OF TITERS OF RETROVIRAL PARTICLES EXPRESSING DIFFERENT ENV PROTEINS ON VARIOUS CELL LINES

	Env protein		
Target Cell	*L1*	*FeLV-A*	*FeLV-C*
143B	$8.4 \pm 4.6 \times 10^3$	$2 \pm 2 \times 10^1$	$2.4 \pm 1.7 \times 10^5$
293T	$1.6 \pm 0.8 \times 10^5$	$1.3 \pm 0.5 \times 10^4$	$1.3 \pm 0.8 \times 10^5$
293	$2 \pm 0 \times 10^1$	$2.7 \pm 0.4 \times 10^3$	$3.4 \pm 1.0 \times 10^4$
A498	<10	<10	$3.0 \pm 1.9 \times 10^3$
DLD-1	$1 \pm 1 \times 10^2$	<10	$8.4 \pm 5.4 \times 10^4$
HeLa	<10	<10	$1.3 \pm 0.8 \times 10^4$
Huh-7	$1 \pm 1 \times 10^1$	$2 \pm 1 \times 10^1$	$4 \pm 1 \times 10^2$
PC-3	<10	<10	$3.4 \pm 0.9 \times 10^3$
RD	<10	<10	$1.2 \pm 0.3 \times 10^5$
TE671	<10	$1 \pm 1 \times 10^2$	$4.3 \pm 0.8 \times 10^5$
W138	$1.7 \pm 0.9 \times 10^2$	$2 \pm 1 \times 10^1$	$4.0 \pm 3.1 \times 10^2$
AH927	$2 \pm 1 \times 10^1$	$5.0 \pm 1.1 \times 10^6$	$1.2 \pm 0.6 \times 10^6$
D17	$3 \pm 1 \times 10^1$	$1.7 \pm 0.7 \times 10^2$	$6.2 \pm 4.8 \times 10^5$
3T3	$3 \pm 3 \times 10^1$	$8 \pm 7 \times 10^1$	$5 \pm 1 \times 10^1$

Supernatants from TELCeB6 cells producing retroviral particles expressing the indicated Env proteins on their surfaces were used to inoculate the

indicated cell lines. Titers are given as average of *lacZ*-colony forming units per ml ± standard deviation ($n \geq 2$ for all cell lines). All acceptors are human except AH927 feline fibroblasts, D17 canine osteosarcoma and 3T3 murine fibroblasts. 143B: Ki-*ras* transformed osteosarcoma; 293: embryonic kidney; 293T: 293 cells expressing SV40 T antigen; A498: kidney; DLD-1: colon; HeLa: cervix; Huh-7: liver; PC-3: prostate; RD: rhabdomyosarcoma; TE671: medulloblastoma; W138: embryonic lung fibroblasts.

TABLE 2. TITERS OF L1 ON KI-*RAS* TRANSFORMED 143B CELLS VS. THE UNTRANSFORMED PARENTAL HOS CELL LINE

ENV	*143B*	*HOS*
L1(concentrated)	2.1×10^5	3.8×10^3 (1.8%)
L1	6.7×10^3	1.8×10^2 (2.7%)
FeLV-B	1.19×10^5	9.3×10^4 (78%)
FeLV-C	1.21×10^5	9.4×10^4 (78%)

Supernatants from TELCeB6 cells producing retroviral particles expressing the indicated Env proteins on their surfaces were used to inoculate the indicated cell lines. Titers are given as *lacZ*-colony forming units per ml and are representative of two separate experiments. Numbers in parentheses indicate percent titer on HOS cells compared with 143B cells.

TABLE 3. SUPERINFECTION INTERFERENCE ASSAYS ON FELV-C-INFECTED 143B CELLS

ENV donor	*Acceptors*	
	143B	143B/FeLV-C
FeLV-C	3.1×10^5	7.8×10^2 (0.25%)
L1	6.7×10^4	6.9×10^4 (100%)

Retroviral particles pseudotyped with the Env proteins noted in the left column were tested for their ability to superinfect 143B cells preinfected with FeLV-C. Titers are *lacZ*-colony forming units per ml. Numbers in parentheses represent percent titer on pre-infected vs. uninfected cells.

TABLE 4. SUPERINFECTION INTERFERENCE ASSAYS ON FELV-A- AND FELV-C-INFECTED 293T CELLS

ENV donor	*Acceptors*		
	293T	293T/FeLV-A	293T/FeLV-C
FeLV-A	5.3×10^3	<5 (0.09%)	8.2×10^3 (150%)
FeLV-C	8.7×10^4	7.2×10^4 (83%)	3×10^1 (0.03%)
L1	1.2×10^5	7.0×10^4 (58%)	3.0×10^4 (25%)

Retroviral particles expressing the indicated Env proteins on their surfaces were tested for their ability to superinfect 293T cells preinfected with FeLV-A or FeLV-C. Titers are *lacZ*-colony forming units per ml. Numbers in parentheses represent percent titer on pre-infected vs. uninfected cells.

TABLE 5. SUPERINFECTION INTERFERENCE ASSAYS ON FELV-B AND AMPHOTROPIC MULV 4070A-INFECTED 293T CELLS

ENV donor	*Acceptors*		
	293T	293T/FeLV-B	293T/4070A
FeLV-B	8.4×10^4	6.2×10^1 (0.07%)	4.7×10^4 (56%)
4070A	1.0×10^5	2.2×10^5 (220%)	2 (0.002%)
L1	1.6×10^5	1.6×10^5 (100%)	7.6×10^4 (47%)

Retroviral particles expressing the indicated Env proteins on their surfaces were tested for their ability to superinfect 293T cells pre-infected with FeLV-B or amphotropic MuLV. Titers are *lacZ*-colony forming units per ml. Numbers in parentheses represent percent titer on pre-infected vs. uninfected cells.

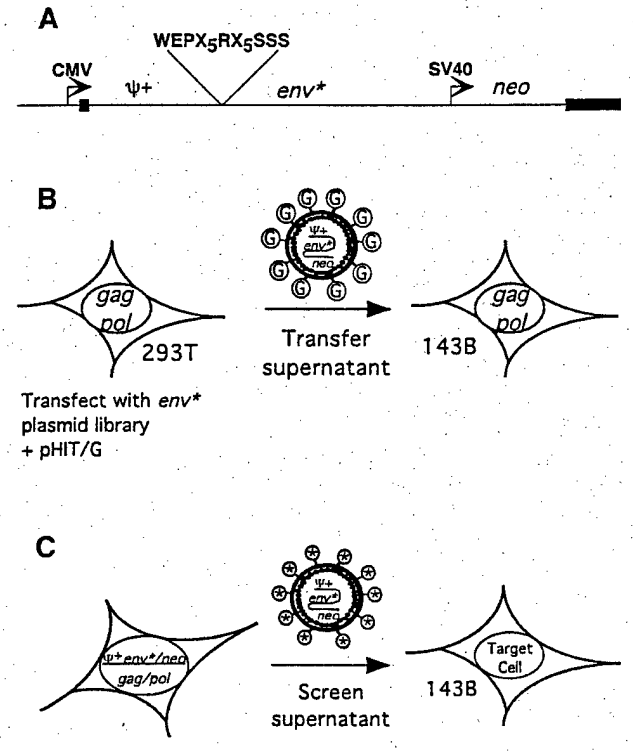
TABLE 6. SUPERINFECTION INTERFERENCE ASSAYS ON 143B CELLS EXPRESSING THE L1 ENV PROTEIN

ENV donor	*Acceptors*	
	143B	143B/L1
L1	1.0×10^4	< 4 (<0.04%)
FeLV-B	4.0×10^5	3.22×10^5 (81%)
FeLV-C	2.74×10^5	2.4×10^5 (88%)
4070A	1.3×10^6	1.4×10^6 (110%)

Retroviral particles expressing the indicated Env proteins on their surfaces were tested for their ability to superinfect 143B cells expressing the L1 Env protein. Titers are *lacZ*-colony forming units per ml. Numbers in parentheses represent percent titer on cells expressing L1 Env vs. cells not expressing L1 Env.

Figure Legend

FIG. 1. Construction and screening of a library of Env* proteins. A: The bicistronic retroviral cassette expressing the library. The amino acid sequence surrounding the cell-targeting region is shown. X denotes randomized amino acids. Black boxes: Moloney MuLV long-terminal repeat, Ψ+: retroviral packaging sequence, CMV: cytomegalovirus promoter, SV40: SV40 promoter, *neo*: G418-resistance marker. B: Creation of a stable retroviral library producer cell line. The plasmid library was transiently transfected into 293T*gagpol* cells along with pHIT-G encoding the VSV-G protein. The supernatant was then used to inoculate 143B*gagpol* cells followed by G418 selection. C: Screening the library. Target cells were inoculated with producer cell supernatant containing viral particles expressing the library of Env* proteins on their surface and packaging the corresponding *env** genes.


A
WEPX5RX5SSS
CMV
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Screen
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Target
Cell
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